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Simultaneous measurement of MMP9 and TIMP1 mRNA in human non small cell lung cancers by multiplex real time RT-PCR

Lisa Simi[a], Matteo Andreani[b], Federico Davini[b], Alberto Janni[b], Mario Pazzagli[a], Mario Serio[c], Claudio Orlando[a,*]

[a] *Department of Clinical Physiopathology, Clinical Biochemistry Unit, University of Florence, viale Pieraccini 6, 50139 Florence, Italy*
[b] *Department of Thoracic Surgery, Azienda Ospedaliera Careggi, Florence, Italy*
[c] *Department of Clinical Physiopathology, Endocrinology Unit, University of Florence, Florence, Italy*

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Summary Extracellular matrix (ECM) homeostasis is strictly maintained by a coordinated balance between the expression of matrix metalloproteinases (MMPs) and their specific inhibitors (TIMPs). Our study was focused on the simultaneous measurement of the expression profile of MMP9 mRNA and its principal inhibitor, TIMP-1, in 100 non small cell lung cancers (NSCLC) and in corresponding adjacent non malignant tissues. The measurement was performed with a multiplex quantitative RT-PCR assay based on TaqMan assay, using two probes labelled with different fluorocromes. We found that both MMP9 and TIMP-1 mRNAs were significantly higher in NSCLC ($P < 0.0001$) in comparison to corresponding controls as well as the MMP9/TIMP-1 ratio ($P = 0.014$). MMP9 and TIMP-1 mRNA expression was highly correlated in cancer samples ($r = 0.73$, $P < 0.0001$). The analysis in the two main histotypes revealed a significant increase of MMP9 mRNA in adenocarcinomas in comparison to normal tissues ($P = 0.006$) but not in squamous cell carcinomas, while TIMP-1 mRNA showed a significative increase both in adenocarcinomas and in squamous cell carcinoma samples ($P = 0.02$ and 0.01, respectively). Both MMP9 and TIMP-1 mRNAs were significantly correlated to lymphnode invasion and cancer stage. Survival analysis revealed that high levels of expression of MMP9 mRNA, but not of TIMP-1, were significantly associated to an unfavourable outcome in NSCLC patients in toto ($P = 0.017$). In addition our results showed that high levels of MMP9 expression are of independent prognostic impact in operable NSCLC. Our data seem to demonstrate a simultaneous and coordinated up-regulation of MMP9 and TIMP-1 expression at the mRNA level in NSCLC, even if this phenomenon seems variable according to the histotype. In addition, the increase of MMP9/TIMP-1 ratio may reflect an unbalance of their production in affected tissues. The increased expression of the two mRNAs, even not necessarily equate their enzymatic activities, seems to parallel a major cancer aggressiveness.

*Corresponding author. Tel.: +39-055-4271-440; fax: +39-055-4271-413.
E-mail address: c.orlando@dfc.unifi.it (C. Orlando).

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1. Introduction

Lung cancer is the commonest cause of cancer death in Europe and United States. The major subtype of lung cancer is non small cell lung cancer (NSCLC) which includes adenocarcinoma, squamous cell carcinoma, and large cell (undifferentiated) carcinoma. The histological type is determinant in establishing prognosis, since subjects with the same stage of disease can show a markedly different progression [1]. Cancer progression involves changes in cell-to-cell adhesion as well as in the interaction between malignant cells and extracellular matrix (ECM). While benign disorders maintain continuous basement membrane, invasive carcinomas have an architectural disorganisation characterised by a discontinuous basement membrane, with zones of matrix loss surrounding the invading tumour cells. Thus, the degradation of basement membrane component appears to be an early event during the metastatic cascade [2]. The ability of malignant cells to invade and metastasize to different tissue compartments was supposed to be strictly associated to the expression of matrix metalloproteinases (MMPs), responsible for the degradation of the principal constituents of the ECM. It appears now evident that most MMPs, as in the case of MMP-9, play an important role even in the early stages of carcinogenesis, prior to basement membrane degradation. MMPs are implicated in cell growth via the activation of mitogenic factors, remodelling, angiogenesis and selection of apoptosis-resistant cells [3—5]. During the pre-malignant stages, stromal and inflammatory cells are responsible for an early secretion of MMPs, while during tumor progression, cancer cells directly control their production [5].

MMPs consist of a family of proteinases with different substrate specificity. In many pathological conditions, modifications of their expression profile causes the lost of balance in the ECM homeostasis. Several studies demonstrated the association between MMP9 and tumour progression in breast [6—9], colorectal [9] and prostate cancer [10]. MMP9, also known as gelatinase B, is a 92 kDa type IV collagenase secreted as inactive glycosylated precursor and subsequently activated by the removal of 73 amino acids at the NH_2 terminus. Because of the ability to degrade the major component of the ECM and basement membrane, its overexpression facilitates the metastatic spread [11]. Growth factors (TGFα, TGFβ, TNFα, EGF), cytokines (interleukin 1 and 4), and hormones (progesterone, retinoids) are responsible for the pre-translational control of MMP9 expression [11,12]. At the post-translational level, all MMPs are under the control of specific tissue inhibitors of metalloproteinases (TIMPs) that bind in proximity of the catalytic domain of MMPs, preventing substrate attachment [13]. TIMPs are not only simple regulators of MMP activity, but they have multifunctional roles that include growth promoting activity [13] and inhibitory effects on angiogenesis [14]. Thus, an overexpression of these inhibitors may be indicative of the stromal cell response to the malignant expansion, but is also probable that tumoral cells themselves directly secrete TIMPs to control their own growth [4,13]. Four members of TIMPs were identified. They inhibit all MMPs, forming non covalent complexes with the active forms. Among them, TIMP-1 has a selective binding with the pro-MMP-9 and is considered the main inhibitor for MMP-9 [13,15]. The detection of MMP9 expression has been performed in lung cancer by immunohistochemistry [1,7,15—19], zymography [9,10,18,20], immunoenzymatic assays [21] and Northern analysis [8,15]. Increased plasma levels of MMP-9 protein were also detected in lung cancer patients [22,23].

Our study was mainly focused on the evaluation of the expression profile of MMP9 and TIMP-1, at the mRNA level, and the clinical relevance of their measurement in lung cancer. To better clarify the reciprocal variation of expression of the two genes, we developed a single-well, single-standard, dual-probe real-time RT-PCR.

2. Materials and methods

2.1. Tissues samples and cell line

The study group consisted of 100 consecutive patients (82 males and 18 females; age range from 46 to 81 years) who underwent surgical excision for NSCLC in the Surgical Department of Careggi Hospital (Florence). NSCLC samples and corresponding non affected tissues, used as normal controls and obtained from the same patient, were immediately snap-frozen in liquid nitrogen. Lung cancer tissues were collected from the invasive periphery of the tumour to exclude more internal area, potentially necrotic. Normal control tissues were taken at 3—5 cm far from cancer during lobectomy and 5—7 cm in pneumonectomy. Comparable tissue samples were processed for routine histological examination. Clinico-pathological features of patients, assessed according to the WHO classification [24] and the TNM staging system, are listed on Table 1.

For RNA extraction, tissues were disrupted by homogenisation in 600 μl of guanidine isothiocyanate-containing lysis Qiagen buffer (QIAGEN, Milan,

Table 1 MMP9 and TIMP-1 mRNA expression in NSCLC and corresponding normal tissues

	Patients	MMP9 mRNA (mean (S.E.))			TIMP-1 mRNA (mean (S.E.))			Ratio MMP9/TIMP-1 (mean (S.E.))		
		Normal	Cancer	P^a	Normal	Cancer	P^a	Normal	Cancer	P^a
Total	100	1.7×10^7 (3.2×10^6)	6.1×10^7 (1.1×10^7)	0.0001	2.9×10^8 (3.6×10^7)	7.6×10^8 (9.9×10^7)	0.00001	0.06 (0.0094)	0.107 (0.017)	0.014
Hystology										
Adenocarcinoma	40	1.6×10^7 (4.0×10^6)	6.0×10^7 (1.4×10^7)	0.006	3.7×10^8 (7.9×10^7)	8.3×10^8 (1.7×10^8)	0.02	0.047 (0.008)	0.084 (0.015)	0.026
Squamous cell	43	2.0×10^7 (5.9×10^6)	5.4×10^7 (1.8×10^7)	0.07	2.6×10^8 (3.5×10^7)	5.8×10^8 (1.4×10^8)	0.015	0.064 (0.014)	0.137 (0.035)	0.06
Adenosquamous	17	1.6×10^7 (8.6×10^6)	8.7×10^7 (3.3×10^7)	0.017	1.7×10^8 (3.7×10^7)	1.1×10^9 (2.4×10^8)	0.002	0.086 (0.044)	0.063 (0.008)	0.5
P^b		0.9	0.9		0.4	0.4		0.07	0.076	
Stage										
1	50	1.3×10^7 (2.8×10^6)	3.4×10^7 (6.5×10^6)	0.007	2.4×10^8 (2.6×10^7)	5.1×10^8 (6.6×10^7)	0.0002	0.049 (0.008)	0.08 (0.012)	0.043
2	18	2.8×10^7 (1.2×10^6)	7.0×10^7 (2.8×10^7)	0.087	3.0×10^8 (1.1×10^8)	6.5×10^8 (2.0×10^8)	0.1	0.111 (0.049)	0.198 (0.616)	0.3
3	32	2.3×10^7 (7.8×10^6)	9.6×10^7 (3.1×10^7)	0.027	3.9×10^8 (9.8×10^7)	9.8×10^8 (2.2×10^8)	0.015	0.057 (0.013)	0.073 (0.013)	0.3
P^b		0.2	0.05		0.2	0.05		0.1	0.003	
T										
T1	26	1.5×10^7 (5.0×10^6)	3.2×10^7 (1.3×10^7)	0.2	2.1×10^8 (3.5×10^7)	2.8×10^8 (5.0×10^7)	0.2	0.057 (0.012)	0.151 (0.042)	0.047
T2	60	2.1×10^7 (5.3×10^6)	7.3×10^7 (1.7×10^7)	0.002	3.1×10^8 (4.5×10^7)	8.4×10^8 (1.2×10^8)	0.0001	0.067 (0.016)	0.08 (0.441)	0.01
T3 + T4	14	1.7×10^7 (6.0×10^6)	5.4×10^7 (3.3×10^7)	0.3	4.0×10^8 (2.0×10^8)	7.2×10^8 (2.4×10^8)	0.3	0.049 (0.016)	0.081 (0.036)	0.4
P^b		0.7	0.3		0.3	0.02		0.8	0.078	
Lymphnodes										
N−	58	1.3×10^7 (2.6×10^6)	3.4×10^7 (5.9×10^6)	0.002	2.5×10^8 (2.7×10^7)	5.2×10^8 (6.3×10^7)	0.0001	0.049 (0.007)	0.086 (0.013)	0.018
N+	42	2.6×10^7 (7.6×10^6)	9.5×10^7 (2.6×10^7)	0.01	3.7×10^8 (8.4×10^7)	9.0×10^8 (1.8×10^8)	0.007	0.081 (0.023)	0.116 (0.027)	0.3
P^b		0.06	0.01		0.1	0.03		0.1	0.3	
Grade										
High	14	2.0×10^7 (7.9×10^6)	2.3×10^7 (8.9×10^6)	0.8	2.5×10^8 (5.0×10^7)	4.1×10^8 (9.8×10^7)	0.2	0.067 (0.019)	0.188 (0.091)	0.2
Medium	68	1.8×10^7 (4.7×10^6)	6.6×10^7 (1.5×10^7)	0.002	2.9×10^8 (4.8×10^7)	7.4×10^8 (1.1×10^8)	0.0002	0.067 (0.014)	0.096 (0.017)	0.15
Low	18	1.6×10^7 (5.3×10^6)	7.3×10^7 (2.8×10^7)	0.06	3.4×10^8 (1.0×10^8)	8.1×10^8 (1.8×10^8)	0.04	0.038 (0.008)	0.079 (0.017)	0.03
P^b		0.9	0.4		0.8	0.3		0.5	0.1	

[a] *t*-test for paired samples.
[b] One-way ANOVA.

Italy) added with β-merchaptoethanol. Total RNA was extracted with QIAshredder and Rneasy MiniKit Qiagen® columns. RNA was then eluted from columns with 50 μl of Rnase free water. Sample were treated with Rnase free DNAse Set QIAGEN® to eliminate DNA. Total RNA concentrations were determined with the GeneQuant spectrophotometer (Pharmacia). RNA extracted from HT1080 fibrosarcoma cell line was used to generate an external reference curve for the simultaneous measurement of both MMP9 and TIMP-1 mRNA expression. This cell line was chosen in a wide panel of human cell lines, since the two mRNAs were expressed exactly at the same levels. In fact, in ten consecutive experiments we obtained comparable Ct values for MMP9 and TIMP-1 mRNAs starting from 25 ng total RNA (20.2 ± 0.6 and 20.4 ± 0.6, respectively). The integrity of total RNA was verified in all samples (normal and cancer samples) by evaluating GAPDH mRNA expression with the Pre-Developed TaqMan Assay Reagent, GAPDH endogenous control kit from Applied Biosystems (Foster City, CA, USA).

2.2. Quantification of MMP9 and TIMP-1 mRNA

The measurement of MMP9 and TIMP-1 mRNA was performed by using a multiplex quantitative real-time RT-PCR method, based on TaqMan™ technology. Probe and primers were selected by the proprietary software ''Primer Express'' (PE Applied Biosystems). For the detection of MMP9 mRNA the following probe and primers were chosen (NCBI accession code: AF148064): probe 984, 5′-TAC CGC TAT GGT TAC ACT CGG GTG GC-3′, labelled with FAM, which hybridises on exon 2; forward primer 130, 5′-CCT GGA GAC CTG AGA ACC AAT C-3′ located on exon 1 and reverse primer 1013, 5′-GAT TTC GAC TCT CCA CGC ATC-3′ located on exon 2. For the detection of TIMP-1 mRNA we selected the specific probe 66: 5′-CCA GAG AAC CCA CCA TGG CCC C-3′, labelled with VIC, which hybridises on the exon 1 and exon 2 junction region; forward primer 47, 5′-TCC AGC GCC CAG AGA GAC-3′, located on exon 1 and reverse primer 167, 5′-AAC AGG ATG CCA GAA GCC AG-3′ located on exon 2 (NCBI accession codes: L47357 and L47361). All the enzymes and reagent used for the reverse-transcription and PCR reaction were provided by PE Applied Biosystems (Milan, Italy). Four hundred nanograms of total RNA were reverse-transcribed in 80 μl of final volume in a reaction mixture containing 10 μl TaqMan RT buffer 1×, 5.5 mM $MgCl_2$, 500 μM each dNTPs, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitors and 1.25 U/μl MultiScribe reverse transcriptase. The profile of the one-step reverse transcription reaction was 10′at 25 °C, 30′ at 48 °C and 2′ at 95 °C. The PCR reaction was performed in 25 ng cDNA, in a reaction mix containing 300 nM of forward primer and 900 nM of reverse primer (for both genes), 12.5 μl Universal Master Mix and 200 nM of each fluorescent probe. The two probes were added in all reaction wells for the simultaneous measurement of the two target genes. Plates were treated 2′ at 50 °C, 10′ at 95 °C and then submitted to 40 cycles of amplification at 95 °C for 15 s, 60 °C for 60 s in the ABI Prism 7700 Sequence Detector PE Applied Biosystems. Standard curves for MMP9 and TIMP-1 mRNAs consisted of serial 1/10 dilutions from 2.5×10^7 to 2.5×10^2 fg HT1080 total RNA. Results were expressed as fg of HT1080 RNA/μg total RNA.

2.3. Statistical analyses

Statistical analysis was carried out using the SPSS software package (SPSS INC, Chicago, IL). Statistical differences between groups were assessed by *t*-test analysis. For analysis of follow-up data, life table curves were calculated using Kaplan-Meier method and survival distribution were compared by log-rank statistics. The primary end point was cancer-related survival, as measured from the date of surgery to the time of last follow-up or cancer-related death. The joint effects with already recognised prognostically relevant variables were examined via Cox proportional hazard analysis. pT-status, pN-status, tumor histology and tumor grading were entered stepwise forward into the model to test these covariables for possible joint effects with high/low levels of MMP9 mRNA expression. Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Real-time detection of MMP9 and TIMP-1 mRNAs

The intra-assay variability of our methods, determined in 10 replicates of a cancer sample assayed in a single experiment and expressed in terms of coefficient of variation of Ct value, was 1.6% for MMP9 and 1.3% for TIMP-1. The inter-assay variability, obtained by 13 repetitions of the same sample in different experiments, was 3.0% for MMP9 and 3.9% for TIMP-1.

MMP9 and TIMP-1 mRNAs were expressed in all cancers and normal tissues. As shown in Table 1, the expression of MMP9 and TIMP-1 mRNAs were

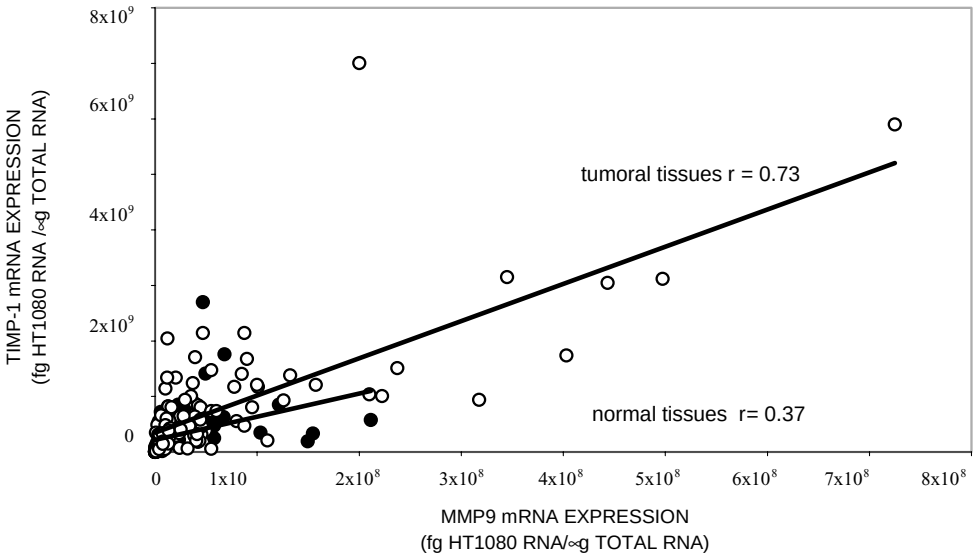

Fig. 1 Linear relationship between MMP9 and TIMP-1 mRNAs in NSCLC (white dots) and in their corresponding normal control tissues (black dots).

significantly higher ($P = 0.0001$ and 0.00001, respectively) in lung cancers than in corresponding normal tissues. The levels of MMP9 mRNA in lung cancers were positively related to tumor stage ($P = 0.05$) and lymphnode status ($P = 0.01$), whereas no statistical difference was detected on the basis of tumor size and differentiation. The expression of TIMP-1 mRNA in lung cancer showed a correlation with stage disease ($P = 0.05$), tumor size ($P = 0.02$) and lymphnode involvement ($P = 0.03$), but not with cancer differentiation. In addition, we found a weakly correlation between MMP9 and TIMP-1 mRNA expression in normal samples ($r = 0.37$, $P < 0.001$), whereas this relationship was more evident in cancer samples ($r = 0.73$, $P < 0.00001$) (Fig. 1). Finally, we evaluated the variation of MMP9/TIMP-1 ratio in normal and cancer tissues, as an index of reciprocal changes of the expression of the two genes. We found that the ratio was significantly higher ($P = 0.014$) in cancer tissues (0.107 ± 0.017) than in corresponding normal tissues (0.06 ± 0.01).

When results were evaluated according to cancer histotype, we found that in adenocarcinomas MMP9 mRNA levels were significantly higher ($P = 0.006$) in cancer tissues than in corresponding normal samples, whereas in the squamous group the difference between normal and tumoral samples was not significant. Conversely, for TIMP-1 mRNA expression, the difference was statistically significant both for squamous cell carcinomas ($P = 0.01$) and for adenocarcinomas ($P = 0.02$). In the mixed group of adeno-squamous cancers, both MMP9 and TIMP-1 mRNAs were significantly higher in cancers ($P = 0.017$ and 0.002, respectively). Finally, we found a significant increment of the MMP9/TIMP-1 ratio in adenocarcinomas ($P = 0.026$) but not in squamous cell carcinomas in comparison to their normal counterparts. In adeno-squamous group the ratio was not significantly different in paired tissues.

3.2. Survival analyses

The median follow-up duration was 25 months (range = 2−43 months). Patients were stratified according to the median levels of MMP9 and TIMP-1 mRNA expression (2.2×10^7 and 4.2×10^8 fg of HT1080 RNA/μg total RNA, respectively). Survival analysis of cancer-related death was obtained by comparing patients with high expression ($\geq$median value) versus patients with low expression ($<$median value). According to this division, we found a significant correlation between high expression of MMP9 mRNA and unfavourable outcome (log-rank test, $P = 0.017$) (see Fig. 2). This difference was not observed for TIMP-1 expression levels ($P = 0.06$). Interestingly, separate Kaplan-Meyer analyses of patients classified on the basis of histotype (adenocarcinomas versus squamous/adenosquamous cell carcinomas) have shown that high MMP9 expression was significantly correlated with adverse outcome in the group of squamous/adenosquamous cell carcinomas ($P = 0.001$) but not in adenocarcinomas ($P = 0.5$).

Table 2 Univariate and multivariate analysis of MMP-9 prognostic value in 100 NSCLC

Risk factor	Univariate analysis *P* value[a]	Multivariate analysis[b]		
		Relative risk	95% Cl	*P* value
pT status	0.030	1.641	0.77—3.51	0.202
pN status	0.010	1.129	0.26—4.81	0.870
Tumor stage	0.001	1.823	0.78—4.26	0.166
High MMP9 mRNA expression[c]	0.022	2.833	1.16—6.91	0.022

[a] *P*-values of univariate analyses were determined by log-rank test.
[b] Stepwise multivariate analysis was performed using the Cox proportional-hazard model.
[c] Samples were stratified according to the median value of the 100 NSCLC (high expression = MMP-9 mRNA $\geq 2.2 \times 10^7$ fg HT1080 RNA/μg total RNA; low expression = MMP-9 mRNA $< 2.2 \times 10^7$ fg HT1080 RNA/μg total RNA).

A multivariate analysis was performed to evaluate whether the correlation between high MMP9 expressing and shortened cancer-related survival resulted from an association of MMP9 expression with other prognostically relevant factor or whether it could maintain its prognostic value. High MMP9 expression, pT status, pN status and tumor stage were tested for possible prognostic joint effects. This Cox regression analysis demonstrated that high MMP9 expression was a significant and independent prognostic parameter for shortened cancer-related survival in patients with NSCLC ($P = 0.022$). The relative risk for cancer related death was 2.8-fold increased in case of MMP9 expression higher than 2.2×10^7 fg of HT1080 RNA/μg total RNA compared to low expressing tumours (95% Cl = 1.16—6.91). Comparison of the relative risks of high MMP9 mRNA expression, pT status, pN status and tumor grading showed that MMP9 expression was the strongest prognostic parameter (Table 2). A similar trend was observed when we repeated the univariate/multivariate analysis in the group of squamous/adenosquamous cell carcinomas ($n = 60$). Once again MMP-9 mRNA expression maintained its significance as independent prognostic parameter for shortened cancer-related survival ($P = 0.041$), with a relative risk of 3.4% (95% Cl = 1.05—11.32) (Table 3). No significance was found in the group of patients with adenocarcinoma ($n = 40$, data not shown).

4. Discussion

The key role of TIMPs in exerting their control on the ECM has prompted investigations in the development of competitive, reversible and broad spectrum synthetic MMP-inhibitors. Even if preclinical studies showed compelling results, the following human clinical trials have been disappointing [3]. However, clinical trials with MMP inhibitors in NSCL cancers were performed only in patients in advanced stage and were irrespective of the analysis of the expression patterns of the entire family of MMPs [25—27]. As recently stated by Bonomi [28], a combined therapy with MMP-inhibitors agents should be limited to patients with MMP overexpression and pharmacological treatments should

Table 3 Univariate and multivariate analysis of MMP-9 prognostic value in squamous and adenosquamous cell carcinomas ($n = 60$)

Risk factor	Univariate analysis *P* value[a]	Multivariate analysis[b]		
		Relative risk	95% Cl	*P* value
pT status	0.640	1.042	0.36—3.04	0.939
pN status	0.059	0.987	0.13—7.61	0.990
Tumor stage	0.032	1.939	0.64—5.91	0.244
High MMP9 mRNA expression[c]	0.018	3.451	1.05—11.32	0.041

[a] *P*-values of univariate analyses were determined by log-rank test.
[b] Stepwise multivariate analysis was performed using the Cox proportional-hazard model.
[c] Samples were stratified according to the median value of the 100 NSCLC (high expression = MMP-9 mRNA $\geq 2.2 \times 10^7$ fg HT1080 RNA/μg total RNA; low expression = MMP-9 mRNA $< 2.2 \times 10^7$ fg HT1080 RNA/μg total RNA).

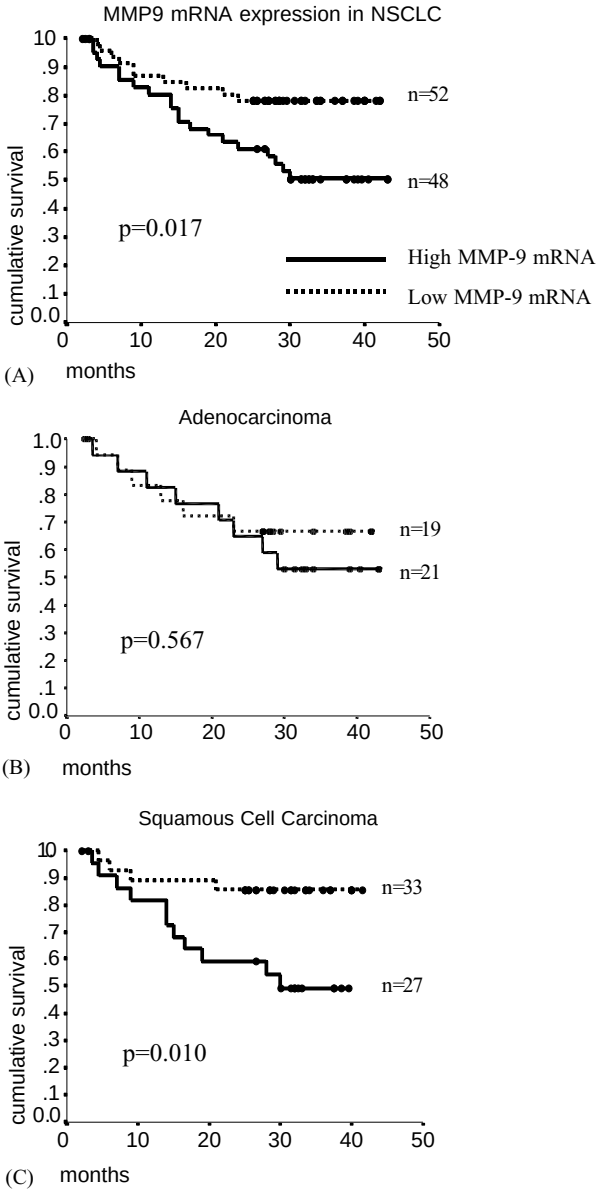

Fig. 2 Cumulative cancer-related survival in patients with high MMP-9 mRNA expression ($\geq 2.2 \times 10^7$ fg of HT1080 RNA/μg total RNA) compared to patients with low MMP-9 mRNA expression ($< 2.2 \times 10^7$ fg of HT1080 RNA/μg total RNA) in: (A) 100 NSCLC; (B) 40 adenocarcinomas; and (C) 60 squamous/adenosquamous cell lung carcinomas. Survival distributions were calculated using Kaplan-Meier method and compared by log-rank analysis.

be tailored accordingly. For this type of treatment, the measurement of expression of the target MMP in tumor, in comparison to the adjacent non cancer tissue, may identify patients eligible for a specific MMP inhibitor therapy [19]. Even if an increased MMP mRNA expression may not be associated to an increased enzymatic activity, because of the necessity of the activation of MMP latent forms, an alteration of MMPs and TIMPs expression in cancer tissues could reflect an altered balance in the control of ECM, which is always a feature of malignant disease [5]. For this reason, our study was focused on the simultaneous measurement of MMP9 and TIMP-1 mRNA expression in NSCLC. Information about the balance between the expression of these genes may be relevant for their application as prognostic factor and in selective cancer therapy. For this specific purpose, the development of a duplex quantitative RT-PCR assay, based on the use of a single external standard, appeared particularly relevant for the absolute comparison of the two mRNAs, which are expected to have a coordinated expression in human tumours. The use of a duplex approach eliminates the expected variability between separated assays, while the use of a single standard provides a tool for the accurate comparison between the expression of two genes.

Our data revealed that at the mRNA level, TIMP-1 is more expressed than MMP9 (about 10-fold) in lung cancers as well as in corresponding normal tissues. The expression of MMP9 and TIMP-1 mRNAs was upregulated in lung cancers in comparison to their control tissues. In addition, we found that the MMP9/TIMP-1 ratio was significantly increased in cancers in comparison to normal tissues, reflecting an unbalancing of their production. When we analysed results separately, in the two main NSCLC histotypes, a different expression profile was evident. In lung adenocarcinomas MMP9 and TIMP-1 mRNAs were significantly higher than in corresponding normal tissues. However, in these tumours, the increase of MMP9 expression, potentially connected with the invasive mechanisms of cancer, is not accompanied by a parallel increase of the expression of its inhibiting partner, TIMP-1. This trend is reflected by the significant increase of the MMP9/TIMP-1 ratio in cancer tissues. Conversely, in squamous cell carcinomas, MMP-9 mRNA expression was not significantly up-regulated, whereas TIMP-1 was found significantly increased in cancer tissues. The increase of mRNA expression of TIMP-1 was able to counteract any modification of MMP9 expression, as demonstrated by the lack of significant variations of MMP9/TIMP-1 ratio in this group. The reason of this histological-dependent expression profile of two genes may be put in relation to the major biological aggressiveness of adenocarcinomas in comparison to squamous cell carcinomas. Finally, in the mixed adeno-squamous cancer group, MMP9 and TIMP-1 were significantly increased in comparison to normal tissues, without any modification of their ratio. In addition MMP9 and TIMP-1 mRNA expression appeared correlated both in normal and cancer tissues. In particular, the high relationship found in lung cancers seems

to confirm that the mechanisms regulating their gene expression are highly coordinated, so that the up-regulation of MMP9 expression is accompanied by a proportional increase of TIMP-1 expression.

Survival analysis of cancer-related death revealed that high levels of expression of MMP9 mRNA, but not of TIMP-1, were significantly associated to an unfavourable outcome in NSCLC patients. The division of our patients according to their histotype clearly demonstrated that the predictivity of MMP9 mRNA expression was effectively significant in squamous/adenosquamous cell carcinomas ($P = 0.01$) but not in adenocarcinomas ($P = 0.5$). In addition, our results showed that high levels of MMP9 expression are of independent prognostic impact in operable NSCLC. A similar trend was observed when we repeated the univariate/multivariate analysis in the group of squamous/adenosquamous cell carcinomas: MMP-9 mRNA expression maintained its significance as independent prognostic parameter for shortened cancer-related survival. No significance was found in the group of patients with adenocarcinoma.

These data, obtained for the first time in quantitative real-time RT-PCR approaches, are in agreement with the recent data, obtained with immunohistochemical staining for MMP-9, showing an association between its expression and patient survival [1,19,29,30]. In addition, we also observed that MMP9 expression represents an independent prognostic marker, as recently stated [19]. However, in contrast to this study, we found a good correlation with some of the commonest clinical features in lung cancer patients. Both MMP9 and TIMP-1 expression was significantly related to lymphnode status. A significant increase of their expression was also evident in advanced tumor stage, as well as in tumours with increased size. No significant relation with cancer differentiation was found.

In conclusion our data seem to demonstrate a simultaneous and coordinated up-regulation of MMP9 and TIMP-1 expression at the mRNA level, in NSCLC, even if this phenomenon seems variable according to the histotypes. The increased expression of the two mRNAs, even if not necessarily reflected by an increase of their enzymatic activity, seems to be connected to a major cancer aggressiveness, as demonstrated by their significant correlation with lymphnode involvement and advanced tumor stage. In addition for the first time, our data seem to indicate a prevalent prognostic relevance of MMP9 in squamous/adenosquamous cell carcinomas, whereas in adenocarcinomas this parameter seems of no clinical value. Future studies, based on the simultaneous evaluation of several MMPs in the same patients, should elucidate the profile of their expression and their clinical relevance.

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